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An Evaluation of the Effectiveness of Azelaic Acid As a Depigmenting and Chemotherapeutic Agent

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In the past five years, it has been reported that certain dicarboxylic acids (C_8–C_{13}) and azelaic acid (C_9) (AZA), in particular, have a remarkable effect in the management of lentigo maligna, human malignant melanoma, and certain disorders of hyperpigmentation. Preclinical trials, therefore, were undertaken in order to evaluate the effectiveness of AZA as a depigmenting agent and as a chemotherapeutic agent. Twenty-seven uniformly black pigmented guinea pigs were given topical applications of various concentrations (3, 5, 10, 15, and 20%) of AZA preparations for 8 weeks, and their effects on the melanocytes of epilated skin of the backs and the nonepilated ears of guinea pigs were compared to the effects of well-known depigmenting agents. Whereas 4-isopropylcatechol, monobenzylether of hydroquinone, monoethylether of hydroquinone, hydroquinone, and 4-hydroxyanisole were found to be selectively cytotoxic to melanocytes in black-skinned guinea pigs, AZA has little or no visually recognizable effect on melanocytes in these animals. The therapeutic effect of local s.c. injections of various concentrations of AZA preparations on the development of s.c. implanted B-16 melanoma tumor was evaluated in 96 C57BL/6J mice. In addition, 31 BDF_1 mice, implanted i.p. with B-16 melanoma tumor, were used to assess the effect of 100–500 mg/kg concentrations of AZA administered i.p. In both studies, AZA revealed no significant tumoristatic or tumoricidal effect on the size, color, and growth of melanoma. The effect of AZA was also evaluated on S-91A (melanotic or pigmented) and S-91B (amelanotic) human melanoma cells in culture. Low concentrations (10^{-5} and 10^{-3} M) of AZA had no inhibitory effect on the growth of these cells. Only at higher concentrations (>10^{-3} M) was a cytotoxic effect on cell viability observed. These observations indicate AZA is not selectively cytotoxic to normal and proliferative melanocytes and has no apparent inhibitory effect on the formative process of melanin pigmentation.

In 1978, Nazzaro-Porro et al proposed a specific, causative relationship between the fungal infection and hypopigmentation in tinea (pityriasis) versicolor. They reported certain dicarboxylic acids (C_8–C_{13}) and AZA (a C_9 dicarboxylic acid), in particular, are competitive inhibitors of tryosinase in vitro [1,2] and exhibit pronounced beneficial effects in vivo on the treatment of hyperpigmentary disorders such as chloasma (melasma), toxic melanoderma, and poikiloderma of Civatte [3]. They also observed azelaic acid (AZA) caused damage to normal melanocytes [2,3]. In studies on mice with transplanted Harding-Passey melanoma, they further reported the i.p., s.c., and oral administration of C_9, C_{12}, and C_{14} dicarboxylic acids significantly retarded the tumor growth and prolonged the survival time of tumor-bearing mice [2,3]. Histologic and electron microscopic examination revealed lipid degeneration of malignant melanocytes and diminished metabolic activity as expressed by poorly developed Golgi apparatus, a reduced number of hypopigmented melanosomes, and associated membrane changes. Furthermore, they reported a striking therapeutic effect in lentigo maligna with clinical and histologic resolution of lesions, when a topical preparation containing 15% or 20% AZA was applied to the lesions; a progressive disappearance of abnormal melanocytes at the basal and suprabasal layers was recorded, and the abnormal melanocytes were observed to be replaced by "normal melanocytes" [2,4,5]. In addition, clinical regression in human malignant melanoma was also seen when AZA was applied topically and given orally. Twenty-three patients with clinically diagnosed malignant melanoma were treated with topical cream (20%) and oral (10–15 g daily) AZA for 1–12 weeks. Clinical improvements were observed in all patients. AZA arrested growth and caused flattening and regression of the size and the advancing edges of the tumor [6]. Recently, Pehamberger et al also reported AZA significantly reduced the proliferation of two melanoma cell lines (CRL 1424-human and CCL 53.1-mouse) but had no adverse effects on cell viability [7]. The review by Breathnach et al [3] provides additional pertinent references to the various properties of AZA.

In vitro and in vivo studies were, therefore, carried out to evaluate these apparent selective cytotoxic and depigmenting properties of AZA and its potential value as a chemotherapeutic agent. In black pigmented guinea pigs, the depigmenting effects of various concentrations of AZA preparations were compared with the effects of the known depigmenting agents: 4-isopropylcatechol (4-IPC), hydroquinone (HQ), 4-hydroxyanisole (4-OHA), monobenzylether of hydroquinone (MBEHQ), and monoethylether of hydroquinone (MEEHQ). The effect of AZA as a chemotherapeutic agent was evaluated in mice bearing subcutaneous B-16 melanoma tumors as well as in mice bearing i.p. implanted B-16 melanoma tumors. The in vitro effect of AZA on the growth and survival of S-91A (melanotic) and S-91B (amelanotic) melanoma cells was also evaluated.

MATERIALS AND METHODS

Cutaneous Depigmentation with AZA and Other Known Depigmenting Agents

Twenty-seven uniformly pigmented black-skinned adult (600 ± 100 g) guinea pigs (Elmhill Breeding Laboratories, Chelmsford, Massachusetts) were used to evaluate the effectiveness of AZA as a depigmenting agent. Reagent grade AZA was purchased from the Eastman Kodak

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Abbreviations:
AZA: azelaic acid (nonanedoic acid)
dopa: 3,4-dihydroxyphenylalanine
HQ: hydroquinone
4-IPC: 4-isopropylcatechol
4-OHA: 4-hydroxyanisole
MBEHQ: monobenzylether of hydroquinone
MEEHQ: monoethylether of hydroquinone

Company, Rochester, New York. Preparations of creams containing 3, 5, 10, 15, and 20% AZA were applied to the wax-epilated skin of the backs and the nonepilated skin of the ears of guinea pigs once daily for a period up to 8 weeks. The 3, 5, 10, and 20% AZA creams were made in an acid mantle cream base, at pH 5.8–6.0, containing glycerol monostearate and aluminum acetate 18%, spermaceti 4%, sulfonated caster oil 4%, acetylated lanolin (modulen) 7%, emulsifier and stabilizer, isopropyl palmitate (deltyl extra) 3%, glycerol 3%, lactic acid 0.25%, H_2O 60%, and preservatives (antioxidants) 0.15%. The 3, 5, 10, and 20% AZA creams in acid mantle base were applied daily (6 days/week) for 6 weeks (45 days) to 20 animals divided into 4 groups. The 15% AZA cream was made in the following base: amerchol 4.3%, olive oil 2.9%; glyceryl monostearate 5.0%, cetyl alcohol 2.4%, stearic acid 2.4%, triethanolamine 0.9%, propylene glycol 2.1%, and distilled water 65%. A group of 8 animals received 15% AZA cream for 8 weeks. In addition, 2 guinea pigs received topical application of a 15% solution of AZA in 95% ethanol to ascertain the contact irritation effect of this decarboxylic acid. Each guinea pig had at least a 56 cm^2 area for product application. Four microliters of AZA preparation were applied to each cm^2 area of the test site. The depigmenting activity of topical AZA was compared to the known depigmenting agents: 3% 4-IPC, 3% 4-OHA, 3% MBEHQ, 3% MEEHQ, and 3% HQ. These chemicals were purchased either from Sigma Chemical Co., St. Louis, Missouri, or from Aldrich Chemical Co. Milwaukee, Wisconsin. The chemicals were applied to the epilated backs of pigmented guinea pigs in a cream form using the same vehicle (acid mantle base) as described above. Controls to compare the morphologic alterations of melanocytes included epilated, untreated skin and vehicle-treated skin (cream base).

Cutaneous depigmentation is maximal by the end of 6 weeks. The degree of cutaneous depigmentation was, therefore, evaluated weekly for up to 8 weeks according to the following criteria: 0 = none; ± = uncertain; + = speckles of depigmentation, weak reaction; ++ = mild depigmentation with some foci of normal skin color; +++ = moderately strong depigmentation with uniform loss of skin color; ++++ = white skin exhibiting total (strong) depigmentation with almost a total loss of dopa-positive melanocytes. Unless otherwise indicated, the term *control* refers to biopsy sites obtained from the epilated skin treated with either of the 2 bases used for preparing depigmenting creams. The cream bases devoid of depigmenting chemicals (e.g., AZA, 4-IPC, 4-OHA, etc.) were applied daily, 6 days per week for a period of 8 weeks. Control biopsies were obtained at various time intervals on those days when AZA-treated biopsies were needed for evaluating the morphologic and numerical alterations in melanocytes. Thiersch biopsies, approximately 1 cm^2 from the product-treated test sites and the control sites, were obtained at 2-week intervals up to 6 weeks and processed for melanocyte counts as described by Szabo [8] and Bleehan et al [9]. The biopsies were assessed for: (1) routine histology (hematoxylin-eosin staining), (2) histochemistry using 3,4-dihydroxyphenylalanine (dopa) incubation to determine the numerical count of melanocytes and evidence of alterations in the morphology of melanocytes, and (3) ultrastructural studies. Electron microscopic studies were carried out according to the method outlined by Jimbow et al [10].

Chemotherapy of B-16 Mouse Melanoma

The effect of various concentrations of AZA on the survival of B-16 melanoma-bearing mice was evaluated using adult male (22–25 g) BDF_1 mice (Jackson Laboratories, Bar Harbor, Maine). The protocol established by the U.S. National Cancer Institute, Bethesda, Maryland, for the testing of chemical agents against animal tumors was followed [11,12]. Because of the limitations imposed by the availability of pure AZA and the required number of animals, the effect of AZA on the growth of B-16 melanoma tumor cell was examined in 2 phases and over a period of 9 months. In the first phase, mice of Group 1 received moderately high concentrations of buffered AZA (200–400 mg/kg) at pH 7.4. In the second phase, mice of Group 2 received low concentrations of buffered AZA (100 mg/kg at pH 7.4), and mice of Group 3 received buffered AZA at high concentration (500 mg/kg at pH 3.2) and low concentrations of AZA (100 mg/kg, pH 3.4). The BDF_1 mice received 0.5 ml i.p. injections of B-16 melanoma-cell homogenate (1.0 g tumor and 10 ml cold balanced salt solution) and then daily injections of 100, 200, 400, and 500 mg/kg AZA solutions. As a control, one group received 0.5 ml tumor injection followed by injections of saline solutions with no AZA, and one group received no tumor and just 400 mg/kg of AZA solution. The AZA solutions of 200 and 400 mg/kg were neutralized by NaOH to pH 7.4 resulting in the administration of 97 mg/kg of sodium for every 400 mg/kg of AZA. Non-neutralized solutions of AZA at 100 and 500 mg/kg were also evaluated with appropriate controls. The AZA solutions were administered starting 1 day after the implantation of the tumor and daily injections were carried out for the length of the survival of the mice. The weight of each mouse was taken every fifth day and postmortem examinations (necropsy) were performed after the death of each animal. The median survival times for each group were calculated.

The effect of various concentrations and the varying pH of AZA solutions on the growth of developing tumor was also evaluated in adult male (22–25 g) C57BL/6J mice (Jackson Laboratories). The freshly obtained tumor fragments from mice bearing B-16 melanoma were carefully diced in saline solution and fragments containing approximately 1×10^6 cells were injected s.c. with the aid of a trochar into the right inguinal area. Daily, local and s.c. injections of buffered (pH 5.6–5.2) and unbuffered (pH 3.2–4.0) AZA solutions at 50, 100, 200, 300, and 400 mg/kg were administered starting the day after implantation of the tumor. The unbuffered solutions were made using Krebs-Ringer solution. The weight of the mice was taken weekly, and the tumor weight was calculated from measurement of the size of the tumor using a Vernier caliper and a centimeter scale [11].

Chemotherapy of S-91A and S-91B Melanoma Cell Lines In Vitro

The origin and maintenance of the melanoma cell lines used in this study have been described previously [13,14]. The S-91A and S-91B cell lines represent pigmented (melanotic) and nonpigmented (amelanotic) human melanoma cell lines. Both cell lines have been maintained in McCoy's 5A medium supplemented with 10% fetal calf serum, 100 μg streptomycin/ml, and 100 U of penicillin/ml in an atmosphere of 5% CO_2 humidified air at 37°C. Approximately 1×10^5 cells/well/ml of media for S-91B were plated in Linbro multiwell tissue culture trays and incubated at 37°C for 24 h. When the cells were in the log phase of growth, the old medium was aspirated gently and was replaced with 1 ml of new growth medium containing either of the 4 drugs (4-OHA, 4-IPC, HQ, AZA) at concentrations ranging from 10^{-5} to 10^{-2} M. The solubility of AZA at room temperature is approximately 2.5 g/liter; therefore, AZA could be easily solubilized in the medium at 10^{-5} to 10^{-2} M range. The cells containing the depigmenting agents at these varying concentrations were incubated at 48 h at 37°C. Subsequently, the cells were harvested and counted in a Coulter counter. The cell morphology was observed during and on different days of incubation. For routine purposes, the viability of cells treated with different depigmenting chemicals was assessed using the commonly used trypan blue dye exclusion technique [15], and a visual count of unstained "live" cells based on the assumption the viable cells will exclude trypan blue and the nonviable cells will absorb the dye. Studies on the inhibition of the growth at different concentrations were carried out in triplicate, and the results are expressed as the percentage of inhibition of growth as compared to the control.

RESULTS

Effect of AZA on Normal Melanocytes (Cutaneous Depigmentation)

The effect of various concentrations of AZA on cutaneous depigmentation in comparison with the other known depigmenting agents is shown in Table I. The wax-epilated skin of the backs and the nonepilated skin of the ears of guinea pigs during the 8 weeks of treatment showed an uncertain (slight)

TABLE I. *Comparison of the effects of topical AZA and known depigmenting agents on cutaneous depigmentation in guinea pigs*

AZA conc	No. of guinea pigs	Skin depigmentation at week					
		1	2	3	4	5	6
3%	4	0	±	±	±	+	+
5%	2	0	±	±	±	±	+
10%	7	0	0	0	±	+	+
15%	8	0	0	0	0	0	0
20%	6	0	±	+	+	+	+

Other agents	Skin depigmentation at week				
	1	2	3	4	5
3% HQ[a]	0	±	+	+	++
3% 4-IPC[a]	±	+	++	+++	++++
3% 4-Hydroxyanisole[a]	±	+	++	+++	+++
3% MBEHQ[a]	±	+	++	++	+++
3% MEEHQ[a]	0	±	++	++	+++

Depigmentation: 0 = Absent, ± = uncertain, + = weak, ++ = moderate, +++ = strong, ++++ = total. See also *Materials and Methods*.

[a] Each of these chemicals has been tested on at least 10 animals.

degree of depigmentation (0 to +) in comparison with the known depigmenting agents such as 3% 4-IPC, 3% 4-HQ, 3% 4 OHA, 3% MBEHQ, and 3% MEEHQ, which showed significant depigmentation (up to +4). Topically applied, AZA at 3, 5, 10, 15, and 20% concentrations produced no recognizable cutaneous effects in the first week of treatment. By the end of the second and third week, most of the treated animals exhibited a moderate degree of irritation and desquamation. Higher concentrations of AZA (10–20%) caused an eczematous oozing reaction and was more irritant than the formulation containing a low concentration of AZA. By the end of the third and fourth weeks, there was no evidence of cutaneous depigmentation. The skin of several animals receiving 10, 15, and 20% AZA exhibited evidence of postinflammatory hyperpigmentation. Contrary to this, the skin of animals treated with 4-IPC 3%, 4-hydroxyanisole 3%, MBEHQ 3%, and MEEHQ 3% exhibited a strong degree of depigmentation (Table I); the treated skin sites appeared almost white and amelanotic. By the end of the fifth and sixth weeks, a few newly growing hair shafts distributed randomly at the AZA-treated sites appeared white, indicating little or no effect of AZA on the proliferative melanocytes of the hair bulbs. Contrary to this, skin sites treated with 4-IPC, MBEHQ, 4-OHA, and MEEHQ revealed a significantly higher frequency of leukotrichia. The control skin sites treated for a period of 8 weeks with two different vehicles revealed no irritation reaction; a mild desquamation resulting from the trauma of epilation could be seen in several sites. Microscopic examination of several control skin biopsies obtained from sites treated with 2 vehicles revealed normal-appearing skin very similar to the epilated but nontreated skin, suggesting these 2 vehicles were nonirritant. The incubation of biopsies in the buffered dopa solution at various time intervals up to 6 weeks revealed no significant numerical changes in the number of melanocytes in the AZA-treated skin sites (Table II), whereas skin sites treated with 4-IPC, 4-OHA, MBEHQ, and MEEHQ revealed a total loss of functional melanocytes (Fig 1). The morphology of control skin melanocytes and melanocytes after treatment with 10 and 20% AZA is shown in Fig 1*A*, 1*B*, and 1*C*, respectively. The cells appear active and dendritic, and there is no evidence of the loss of tyrosinase activity as revealed by examination of dopa-incubated biopsies. Examination of the dopa-incubated biopsies by light microscopy revealed no mor-

TABLE II. *Dopa-positive melanocyte count/mm² in guinea-pigs treated topically with 15% AZA cream*

Guinea pig #	Initial		2 Weeks		4 Weeks		6 Weeks	
	Control/treated		Control/treated		Control/treated		Control/treated	
1	515	483	483	489	467	492	480	470
2	492	499	499	486	499	473	505	467
3	464	476	396	460	484	426	416	467
4	486	473	662	531	461	424	464	476
5	424	424	320	378	419	499	460	486
6	426	416	348	540	455	483	489	486
7	419	348	419	403	486	486	492	480
8	502	486	380	374	492	456	470	486
Average ± SD	466 ± 39	451 ± 51	439 ± 83	458 ± 66	470 ± 26	468 ± 29	473 ± 28	477 ± 8

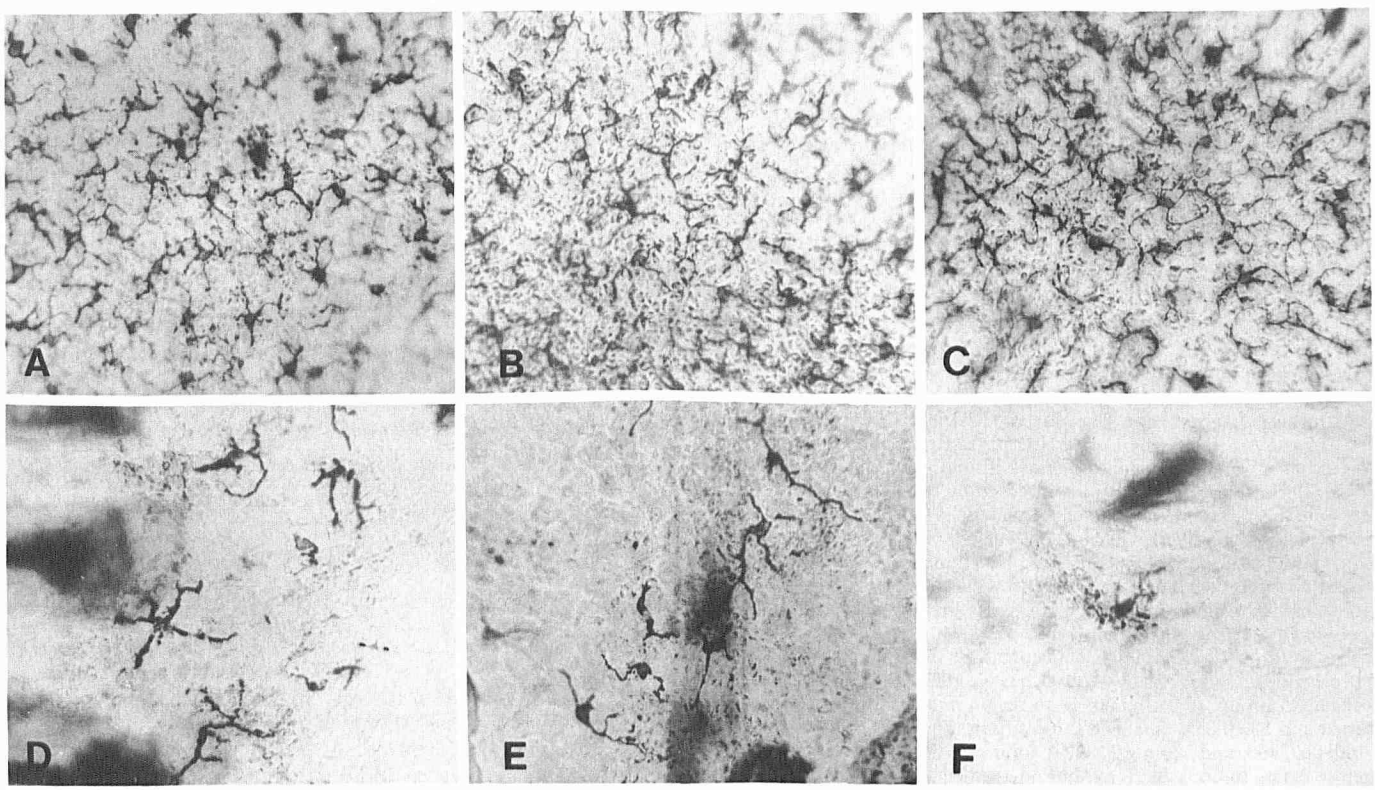

FIG 1. These figures illustrate the in vivo effects of AZA, 4-IPC, MBEHQ, and MMEHQ on guinea-pig melanocytes. *A* = Control, epilated back skin treated with vehicle alone; *B* = 10% AZA; *C* = 20% AZA; *D* = 3% 4-IPC; *E* = 5% MBEHQ; and *F* = 1% MMEHQ (× 550). Pigmented guinea pigs were treated daily, 5 times per week, for 8 weeks. Normal-appearing, dopa-positive dendritic melanocytes can be seen in *A–C*. In *D–F*, progressive loss of melanocytes is seen. A few dopa-positive melanocytes appear hypertropic in *D* and *E* at 5 and 6 weeks of treatment. Eventually by 6–8 weeks, melanocytes degenerate as shown in *F*.

phologic changes in melanocytes treated with AZA, whereas melanocytes treated with the known depigmenting agents 4-IPC, 4-OHA, MBEHQ, and MEEHQ revealed significant degenerative changes with the loss of dendrites and perikaryon. The ultrastructural observations of AZA-treated skin sites (10 and 15% preparations) showed no selective changes in the morphology of the melanocytes. The melanized melanosomes appeared normal, and the other components of melanocytes, such as mitochondria, smooth and rough endoplasmic reticulum, and Golgi apparatus, appeared normal. We found no evidence of abnormal vacuolization or accumulation of lipid droplets as reported by Breathnach et al [16,17]. No myelin-like bodies, which are often seen in degenerating melanocytes [10], were observed; however, in many melanocytes, the mitochondria appeared enlarged in size. Hematoxylin and eosin-stained tissue sections of AZA-treated biopsy specimens revealed a mild hyperplasia of the epidermis. The pigmented cells appeared normal, or in many instances proliferative. The pigment incontinence in the dermis was not seen. The influx of inflammatory cells was minimal at low concentrations. At high concentrations of AZA, a mild inflammation characterized by migration of lymphocytes and polymorphonucleocytes was observed.

Effect of AZA on the Growth of B-16 Melanoma Tumor

The effect of various concentrations of AZA on the median survival time of mice bearing B-16 melanoma tumor i.p. and on the development of s.c. grown tumor is shown in Tables III and IV. AZA showed no beneficial or therapeutic effect on the median survival time of mice bearing intraperitoneal tumor (Table III). Even though statistically not significant, tumor-bearing control mice receiving no AZA survived longer than mice being treated with AZA. Mice of Group 1 receiving moderately high concentrations of buffered AZA (200–400 mg/kg) at pH 7.4 survived as long as the tumor-bearing control animals receiving saline injections without AZA. Mice of Group 2, receiving low concentrations of buffered AZA (100 mg/kg) also showed negligible differences on the medium survival time. There was no evidence of a statistically significant association of survival days between the control group and the AZA-treated group ($n = 10$, $p > 0.05$). On the other hand, mice of Group 3 receiving unbuffered AZA at 100 and 500 mg/kg concentration did not survive long. B-16 melanoma tumor-bearing control mice usually survived 18–21 days. The unbuffered AZA caused irritation, ulceration, and necrosis at the site of injection, and mice died early because of apparent toxic pH effect of AZA. Postmortem examination of the mice revealed in all cases extensive tumor growth in the abdominal cavity with invasion into the thoracic area. In addition, there was no visible change in the color of the tumor.

The effect of AZA on the development of s.c. grown tumor was minimal (Table IV). The buffered solutions of AZA showed no tumoristatic or tumoricidal effects. Although the mean weight and size of B-16 melanoma tumors in mice of Group 2 (100 mg/kg AZA at pH 3.3) and Group 3 (200 and 300 mg/kg AZA at pH 3.5) showed an apparent dimunition in size suggesting AZA was tumoristatic, one should be cautious in interpreting such data. The unbuffered solutions appeared to be irritant as evidenced by the pronounced gnawing at sites adjacent to the tumor. This effect resulted in the loss of tumor mass by the third and fourth weeks in approximately 50% of the mice. When the average weights of the s.c. developing tumor in mice treated with local applications of AZA were compared, it was clear AZA did not inhibit the growth of the tumor. There was a 10- to 15-fold increase in the tumor volume between the first and third weeks. The majority of the animals began dying after 3 weeks of injections, and those that survived continued to show an increase in tumor mass with no evidence of tumor regression. The low average weight often resulted from the mice having destroyed their tumors, thus lowering all averages. Ulcers were evident at the unbuffered AZA injection sites even in non-tumor-bearing mice.

TABLE III. *Median survival time of BDF_1 mice bearing B-16 melanoma tumor intraperitoneally treated with various concentrations of AZA*

Tumor	AZA (mg/kg)	No. of mice	Percent of mice growing tumor	Median survival time in days
Group 1[a]				
No tumor	400	8	0	180.0
+	0	8	100	21.0
+	400	8	100	20.0
+	200	7	100	19.0
Group 2[a]				
+	0	10	100	18.0
+	100	10	100	16.5
Group 3[b]				
+	0	10	100	27.0
+	0	10	100	26.5
+	100	10	100	4.0
+	500	10	100	2.0

[+] Tumor bearing.
[a] Buffered AZA injected i.p. daily; pH 7.4.
[b] Unbuffered AZA injected i.p. daily; pH 3.4.

TABLE IV. *Mean tumor weight and size of developing subcutaneous B-16 melanoma tumor in C57BL/6J mice receiving subcutaneous injections of AZA*

	AZA dose (mg/kg)	pH	No. of mice at 22 days	Mean tumor weight and size at 22 days	
				Weight (mg)	Size (mm^2)
Group 1					
Control	0	7.4	7/8	2849 ± 1209	343 ± 105
AZA	50	6.6	7/8	4301 ± 2004	451 ± 140
AZA	100	6.6	6/8	4391 ± 2385	455 ± 167
AZA	200	5.7	7/8	5706 ± 1752	565 ± 107
Group 2					
Control	0	7.0	8/8	1965 ± 965	268 ± 109
AZA	50	3.4	7/8	3877 ± 2160	440 ± 140
AZA	100	7.4	7/8	2776 ± 776	326 ± 62
AZA	100	3.3	4/8[a]	790 ± 550	130 ± 133
Group 3					
Control	0	7.4	7/8	2859 ± 1559	337 ± 132
AZA	200	3.5	5/8[a]	456 ± 815	88 ± 113
AZA	300	3.6	6/8[a]	1490 ± 1281	213 ± 133
AZA	400	3.4	5/8[a]	0	0
AZA	500	3.2	0/8[a]	0	0

[a] Many mice chewed their skin at the injection sites resulting in gnawing-out of the tumors and over 50% died within 22 days.

The Effect of AZA on S-91A and S-91B Cell Lines

The effect of various concentrations of AZA on the growth of S-91A and S-91B melanoma cell lines is shown in Table V, and its effect in comparison with various concentrations of 4-OHA, 4-IPC, and HQ is shown in Fig 2. At low concentrations, 10^{-5} to 10^{-3} M, AZA was not cytotoxic. At these concentrations, the cells were found to remain attached with a tendency to round. The cells that were attached appeared normal as evidenced by the trypan blue dye exclusion technique. As the concentration of the drug was increased (10^{-2} M) the cells tended to round, swell, and lose their morphology. The cells, soon after contact with 10^{-2} M AZA, became trypan blue positive. The cytotoxic effects of AZA (10^{-2} M) was similar on pigmented and nonpigmented cells. Contrary to this, 4-OHA, 4-IPC, and HQ were cytotoxic at much lower concentrations (10^{-5} to 10^{-4} M), and most of the cells seemed to degenerate with cytotoxicity increased against the pigmented cells. On a molar basis, 4-OHA and 4-IPC were more inhibitory to the growth of melanocytes than was AZA.

DISCUSSION

The initial observations by Nazzaro-Porro et al on AZA provided an interesting explanation of the hypopigmentation so commonly seen in patients with tinea versicolor [1,3]. Their in vitro experiments revealed a causative relationship between the infecting fungus and its metabolic activity generating C_8–C_{13} dicarboxylic acids through the enzymatic oxidation of fatty acids [1,2]. Their subsequent studies with laboratory animals with transplantable Harding-Passey melanoma revealed that daily i.p. or s.c. injections or oral administration of C_9–C_{12} dicarboxylic acids remarkably delayed the tumor growth and prolonged the survival times of tumor-bearing mice [2,3]. These investigators further showed that patients with lentigo maligna, when treated with AZA, responded with progressive reduction in the number of melanocytes and remarkable clinical improvement with return to normal clinical as well as histologic appearance of the skin [2,4,5]. Further clinical trials with AZA revealed this dicarboxylic acid exhibited a therapeutic effect on lentigo maligna as well as a profound effect on patients with invasive melanoma [3,6]. The clinical and histopathologic observations on lentigo maligna treated with 20% topical AZA have been recently supported by Leibl et al [18]. Throughout their studies in humans and in laboratory animals, these investigators have reported negligible effects [2,3,14] on normal cells, and, in general, have supported the view that AZA has no apparent side effects nor is it selectively cytotoxic to normal melanocytes [2–4,6,19]. They believe that AZA acts specifically against hyperactive and abnormal melanocytes with little or no effect on the majority of normal melanocytes. In addition, they have not found an LD_{50} value in mice using doses up to 1000 mg/kg [19].

TABLE V. *Effect of AZA on S-91A and S-91B melanoma cells in culture*

Treatment	Time	Mean no. of melanocytes[a]	
		S-91A pigmented	Percent control
None (control)	Day zero	0.73×10^5	
None (control)	48 h	2.3×10^5	100
10^{-4} M AZA	48 h	2.5×10^5	111
10^{-3} M AZA	48 h	2.1×10^5	83
10^{-2} M AZA	48 h	0.66×10^5	28
		S-91B Nonpigmented	Percent control
None (control)	Day zero	0.46×10^5	
None (control)	48 h	3.5×10^5	100
10^{-4} M AZA	48 h	3.4×10^5	96
10^{-3} M AZA	48 h	3.0×10^5	84
10^{-2} M AZA	48 h	0.89×10^5	14

[a] Each concentration effect was evaluated in triplicate.

While we have not tried to reproduce the interesting findings of these investigators, we have attempted to evaluate the cytotoxic and tumoricidal effect of AZA on normal as well as malignant melanocytes. We have found no evidence AZA functions as a cytotoxic or as a tumoricidal agent in concentrations up to 10^{-3} M.

With several derivatives of HQ (e.g., HQ, MBEHQ, and MEEHQ), several catechol derivatives (e.g., 4-IPC, 4-hydroxy butyl catechol), and several thiol compounds (e.g., mercaptoethanolamine) we have found invariably that a cytotoxic agent to normal melanocytes causes cutaneous depigmentation both in animal and human skin [9,20,21]. These agents are not only cytotoxic to normal, nonproliferative melanocytes, but they equally affect proliferative, hypertrophic, and nonmalignant melanocytes. By visual observations, histochemical staining of dopa-positive melanocytes, and electron-microscopic ultrastructural studies, we have found no evidence that AZA in the 10^{-6} to 10^{-3} M concentration range is selectively cytotoxic to normal or abnormal melanocytes and affects melanocyte morphology and its function (formation, melanization, and transfer of melanosomes). Histochemical studies show both normal and malignant melanocytes are not affected at physiologic concentrations ranging from 10^{-4} to 10^{-3} M of AZA. The perikaryon and the dendritic processes appeared to be normal. In many biopsy specimens, the AZA-treated cells appeared to be proliferative, more dendritic, and had enlarged perikaryon. The ultrastructural studies showed normal-appearing melanosomes, enlarged but intact mitochondria, little or no accumulation of lipid droplets, and no changes in the Golgi apparatus or the rough and smooth endoplasmic reticulum. The data shown in Tables I and II indicate that AZA in various concentrations ranging from 3–20% had no detectable effect on the functional activity of normal pigment-producing cells. It should be realized that in epilated skin, the melanocytes were proliferative and hypertrophic, and while other depigmenting agents shown in Table I selectively affected melanocytes, AZA failed to inhibit or alter the functional state of these active melanocytes. From previous experience with other cutaneous depigmenting and melanoma chemotherapeutic agents, we have not encountered an agent that selectively affects malignant melanocytes but spares the normal melanocytes. On the contrary, phenols, catechols, and thiol compounds not only can selectively destroy normal pigment-producing cells, but will also exhibit cytotoxic effects on malignant melanocytes (unpublished observations). In studying the effects of AZA on 2 melanoma cell lines (CRL 1424-human and CCL 53.1-mouse), the Viennese coinvestigators Leibl et al [18] and Pehamberger et al [7] have recently observed AZA at 1–4 × 10^{-2} M concentration to reduce the proliferation of CRL and CCL melanoma cells but to produce no adverse effects on cell viability. They believed AZA reduces proliferation of melanoma cells due to an inhibition of DNA synthesis. Interestingly, our laboratory studies in progress also

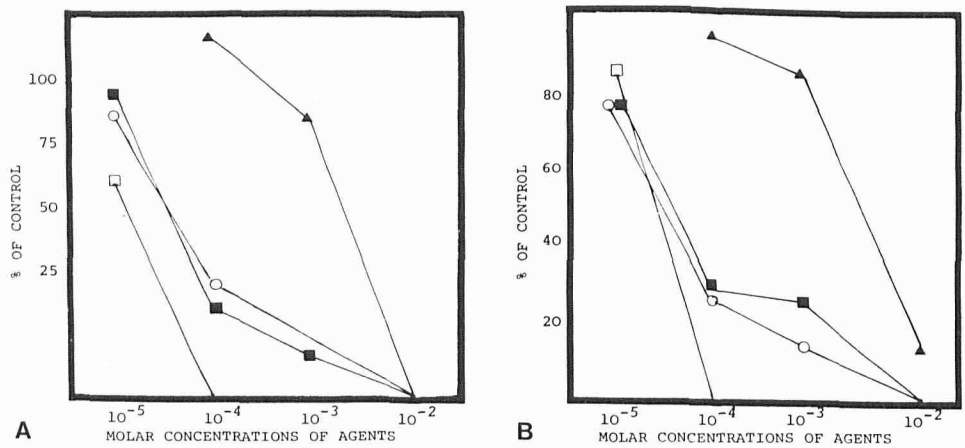

FIG 2. This graph illustrates the effect, as a percentage of the control, of AZA, 4-IPC, HQ, and 4-OHA on the growth of S-91A and S-91B cell lines at 48 h after exposure to various concentrations of the depigmenting agents. On the left side are data pertaining to S-91A pigmented cell line, and on the right side are the data pertaining to S-91B nonpigmented cell line.

indicate that exposure of S-91A pigmented and S-91B nonpigmented melanoma cells to 1×10^{-4} to 1×10^{-3} M concentrations of 4-OHA or 4-IPC resulted in a significant decrease in DNA synthesis as evidenced by the inhibition of [^{3}H]thymidine incorporation. There was, however, no decrease in the de novo synthesis of total cell protein or RNA, suggesting that 4-OHA or 4-IPC, the 2 potent depigmenting agents, also affect the proliferation of melanocytes in a manner similar to AZA as reported by these Viennese investigators [7,18].

Tumor-bearing animals with i.p. or s.c. transplantations of B-16 melanoma revealed no obvious inhibition of tumor growth when treated with AZA. The determination of tumor weight and size in s.c. tumor-bearing mice (Table IV) revealed no consistent tumor inhibition. When AZA was administered in a neutralized pH range, it had no tumoricidal effect. There was, however, some evidence of inhibition when mice bearing s.c. B-16 melanoma tumor received nonbuffered (pH 3.2–3.6) solutions of AZA. These injections appeared to cause localized irritant reactions, many progressing to ulceration. This resulted in the partial destruction of most tumor sites. The decrease in the average tumor weight and size, noted with unbuffered AZA in Table IV, could be attributed to this mechanical removal. Nazzaro-Porro et al have reported their results using AZA as a disodium salt or as a dimethyl ester [2]. We have applied AZA topically and injected AZA both as the disodium salt as well as the nonbuffered acid in varying concentrations and found neither to be inhibitory to tumor growth up to the 10^{-3} M concentration range. Unbuffered AZA preparations, when applied topically or injected intradermally, were highly irritant and caused desquamation, postinflammatory hyperpigmentation in guinea pigs, and ulceration in mice (Table II).

Our data on the cytotoxic effects of AZA on the S-91A (melanotic) and S-91B (amelanotic) melanoma cell lines (Table V) failed to indicate any selective inhibitory effect of AZA at concentrations in the range of 10^{-4} to 10^{-3} M. Higher concentrations (10^{-2} M) appeared to be cytotoxic and inhibited the viability and growth of melanoma cells, but this cytotoxicity was nonspecific owing to the unfavorable growth conditions resulting from perturbations of hydrogen ion concentration.

Nazzaro-Porro and Breathnach and their colleagues have conducted clinical trials using AZA preparations [2–6,22], and some of their clinical photographs, histologic criteria, and mitochondrial enzyme inhibition studies indicate that AZA is cytotoxic to abnormal-appearing, pigment-producing cells. Besides these studies, there is one additional report on lentigo maligna confirming the effects of AZA on hyperactive and abnormal-appearing melanocytes [7]. Willshaw and Rubinstein, in 4 cases of ocular melanoma, have not found AZA to be effective in the treatment of human melanoma. Furthermore, although the AZA (12 g/day) in their study was generally well tolerated, one of their patients developed severe hypokalemia during the treatment [23].

Investigations on the acute toxicity of AZA after oral and i.p. administration, as reported by Mingrone et al [19], indicate AZA is nontoxic and has no teratogenic effects. Our observations on mice, albeit limited in nature, also support these investigators' findings that AZA in the physiologic dose range of 10^{-6} to 10^{-4} M has no apparent toxic effect on the adult animal, but it is toxic at high concentrations, especially when AZA is administered in unbuffered acid form.

The observations reported in this communication raise a few major issues concerning the effects of C_8–C_{12} dicarboxylic acids. First is it possible AZA, the C_9 dicarboxylic acid, has no cytotoxicity to normal melanocytes or atypical malignant melanocytes, and other dicarboxylic acids not investigated by us exhibit the selective inhibitory effect on abnormal melanocytes? Certainly, additional preclinical investigations are warranted to confirm the interesting findings reported by Nazzaro-Porro and other coinvestigators. The effect of buffered and unbuffered AZA must be critically evaluated with respect to pH, concentration, and systemic toxicity. The second issue is whether these saturated dicarboxylic acids (C_8–C_{12}) in their in vivo oxidative metabolism generate metabolites which selectively damage mitochondrial membranes and associated vital enzymes [24,25] or whether AZA significantly reduces proliferation of abnormal cells only due to inhibition of DNA synthesis. Additional clinical and laboratory studies will have to be undertaken to determine the species differences and the specific cytotoxic effect of these saturated medium chain-length dicarboxylic acids on human malignant melanocytes and melanoma cells from other species. Alternatively, it is possible AZA may have a specific effect in humans not seen in the animal models and cell lines employed in this study.

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